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A "LOOP" METHOD FOR COUNTING VIABLE BACTERIA OR BACTERIOPHAGE[1]

By I. N. Asheshov and F. C. Heagy[2]

Abstract

A "loop" method for counting viable bacteria or bacteriophage is described. In this method standard wire loops are used to transfer definite volumes of cultures in two dilution tubes and to spread fluid from the final dilution on an agar plate. Instead of a number of tubes and pipettes that must be laboriously cleaned, plugged, and sterilized, the only requirements are two sterile tubes containing 20 ml. of saline and two wire loops. The loops are sterilized by flaming.

A "loop" method for counting viable bacteria or bacteriophage has been used by the senior author for many years with satisfactory results (1). Its chief advantage is that the only equipment required is two wire loops that are sterilized by flaming—plus the usual dilution tubes and agar plates, of course. The method is being republished because the original description is not generally available.

Method

Principle

Standard wire loops are used to transfer definite volumes of culture to two dilution tubes containing measured amounts of sterile saline or broth. A measured drop of fluid from the final dilution tube is spread on one quadrant of an agar plate by means of one of the standard loops. By plating a range of dilutions and then counting the number of colonies that develop from the most suitable dilution, the number of viable bacteria that were present in the original culture can be calculated.

The same technique is used for making clearing counts of bacteriophages. In this case, the final dilution from the bacteriophage suspension is made into a tube containing about 10^8 sensitive bacteria per milliliter.

Requirements

1. A standard 'small loop'. This is made of 0.3 mm. thick platinum or nichrome wire, the inside diameter being 1.5 mm. This loop, if operated as

[1] *Manuscript received September 18, 1950.*
Contribution from the New York Botanical Garden, New York, N.Y., and the Department of Biochemistry and the Department of Bacteriology and Immunology, University of Western Ontario, London, Ont.
[2] *Formerly Medical Research Fellow, National Research Council, Canada.*

instructed, will take about 0.0010 ml.: the loop after immersion in the liquid is slowly taken out of it, not flat but sidewise. In this case only a thin film will be formed in the opening of the loop.

2. A standard 'big loop'. This is made of 0.5 mm. thick platinum or nichrome wire, the inside diameter being 3 mm. It is bent in a particular manner (Fig. 1) to ensure better holding of the drop and easy spreading. The loop is taken out of the liquid flat, with a smooth jerk, so that an almost hemispherical drop forms in the loop (Fig. 2). This loop will contain about 0.025 ml., which is a suitable amount to spread on one quadrant of an agar plate (Fig. 3). With a little practice drops of uniform size can be obtained.

These two loops can be conveniently made around standard steel gauges. Steel metal drills of 1.5 mm. and 3 mm. diameter are useful substitutes. The best instrument for the purpose is jeweler's pliers for making chain links. A skilled mechanic can easily adjust the proper size of the prongs by polishing away some metal or by adding a steel ring around. Using these pliers it is easy to make a perfect tightly closed loop, essential for holding a standard amount of liquid.

3. Two sterile, plugged Pyrex glass tubes 25 $\times$ 200 mm. containing 20 ml. of saline or broth, for diluting the culture that is being counted.

Calibration of Wire Loops

Each wire loop must be calibrated by the person who uses it. To calibrate a 'big loop', a tube containing a few milliliters of water is accurately weighed, and then the loop is used to remove a standard drop. The tube and contents are weighed again and the difference in weights gives the weight of water in the drop. The procedure is repeated a number of times, and the average volume of fluid per loopful is calculated. To calibrate a 'small loop' the same procedure is followed, except that 10 small loopfuls are removed for each weighing.

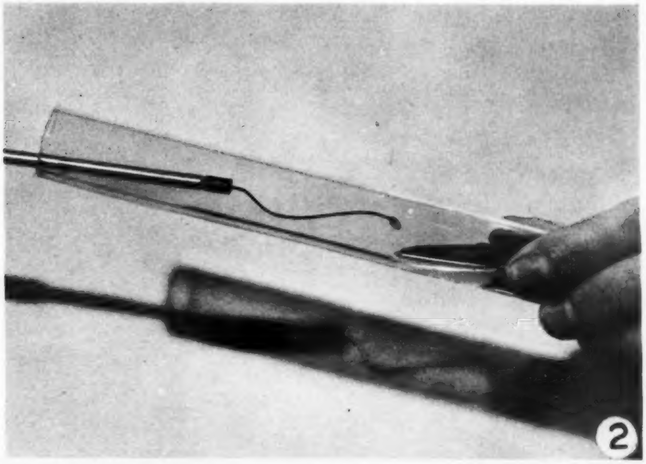
From 20 determinations, one of the authors found that his 'big loop' removed 25.09 ± 1.264 (SD) mgm. of water per loopful (SEM = 0.29 mgm.). For purposes of calculations this big loop was considered to hold 0.025 ml. From 10 weighings he found that his 'small loop' removed 8.9 ± 0.4 (SD) mgm. of water in 10 loopfuls. This is equivalent to 0.89 ± 0.04 (SEM) mgm. per loopful, or 0.89 ± 0.12 (SD). For purposes of calculations the volume 9×10^{-4} ml. was assigned to this small loop.

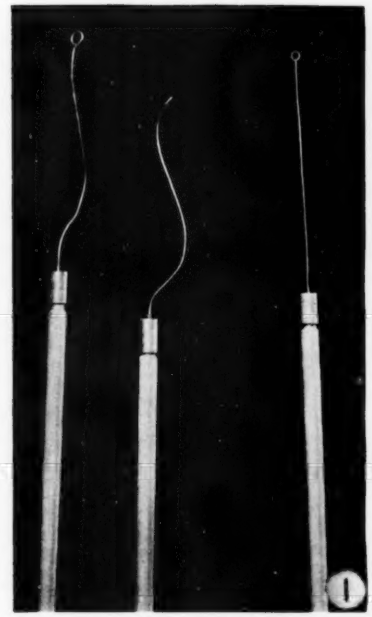
Procedure for Routine Counting

For routine counting, it is convenient to use the following scale of dilutions:

1. 'Small of the big loop' dilution—'SBL'. A big loop of culture is diluted into the first dilution tube. From it a small loop is diluted into the second dilution tube, from which a big loop is plated on agar.

2. 'Big of the big loop' dilution—'BBL.' From the same first dilution tube, which contains a big loop of culture, a big loop is transferred to the second dilution tube, and from it a big loop is plated on agar.





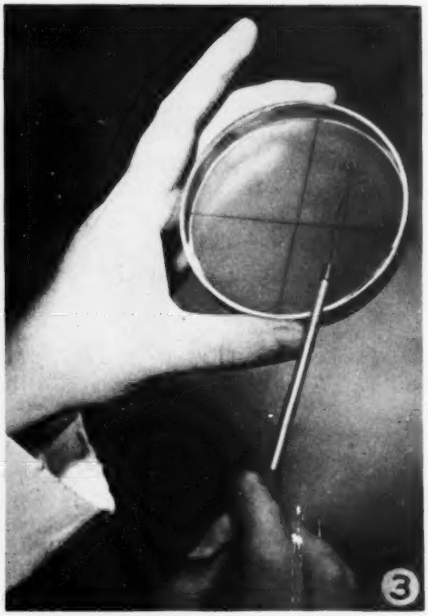


FIG. 1. Standard loops: three-quarters and side views of a 'big loop' (left and center); small loop (right).

FIG. 2. Taking a 'big loop'. The loop is taken out of the liquid flat, with a smooth jerk, so that an almost hemispherical drop forms in the loop.

FIG. 3. Plating. The loop is turned over to deposit the drop of liquid at the top of the quadrant and spread evenly as it flows downward. It is important to arrange the illumination so that the light is reflected from the agar surface.

3. 'Small loop' dilution—'SL.' From the culture, a small loop is transferred to the second dilution tube, and from it a big loop is plated on agar.

4. 'Big loop' dilution—'BL.' From the culture a big loop is transferred to the second dilution tube and from it a big loop is plated on agar.

5. If very few organisms are believed to be present, a 1 : 20 dilution is made by pipetting 2.2 ml. of the culture into the second dilution tube and from it a big loop is plated. For even smaller concentrations, a big loop is plated directly from the original culture.

Calculations

To calculate the original concentrations, the counts per quadrant are multiplied by the appropriate dilutions factors. Using the small loop that holds 9×10^{-4} ml. and the big loop that holds 0.025 ml., the counts are multiplied by the following factors:

For SBL, by 7.2×10^8
" BBL, " 2.5×10^7
" SL, " 8.9×10^5
" BL, " 3.2×10^4

Since there is a ratio of about 1 : 30 between successive dilutions, it is likely that one of the dilutions will give an adequate number of discrete bacterial colonies (or phage clearings). If necessary a higher dilution can be obtained by using a "Small of the small loop' (SSL) dilution for which one starts by diluting a small loop into the first dilution tube, and from it a small loop into the second dilution tube, from which a big loop is plated. All the dilutions are made in the same two tubes, because with a factor of 30 between the various dilutions the error introduced by using the same tubes is not great.

Accuracy

To test the accuracy of the method for counting phage clearings, 40 big loops were plated from a suspension of bacteria containing bacteriophages. One quadrant of one plate was contaminated, and the average phage count of the other 39 quadrants was 161.5 ± 19.2 (SD). In another trial, with different material, 40 big loops had an average count of 248.2 ± 30.2 (SD). In the first example, the theoretical sampling error is $\sqrt{161.5} = \pm 12.7$, and the error due to the method is $\sqrt{19.2^2 - 12.7^2} = \pm 14.4$, or 8.9%, In the second example the error due to the method is $\pm 10.4\%$.

Comments

The error of this method must be considered in relation to the total procedure. As a method for determining counts that may be of the order, say, of 10^4 to 10^{10} per ml. it is a useful procedure. Instead of a number of tubes and pipettes that must be laboriously cleaned, plugged, and sterilized, the only requirements are two sterile tubes containing 20 ml. saline and two wire loops.

The loops are sterilized by flaming. When pipetting small numbers of bacteria, there is considerable error due to bacteria adhering on the wall of the pipette, and with drop methods there may be error due to splashing. Errors of this type are reduced with the loop method, partially offsetting the error in volume measurement.

Frequently the range of expected concentration is limited, and it is not necessary to use the full scale of dilutions: e.g., if the expected concentration is in the range of 10^8 to 10^{10} organisms per ml. the SBL and BBL dilutions will be adequate. It is then feasible to make replicate platings of each dilution and average the counts to reduce the error. However, at such times it is often more convenient and quicker to plate 0.1 ml. volumes with an 0.1 ml. pipette, instead of plating four 0.025 ml. drops with a big loop.

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PARALLEL STUDIES OF COMPLEMENT AND COAGULATION

II. THE RELATIVE EFFECTS OF DICUMAROL[1]

By Paul Boulanger and Christine E. Rice

Abstract

A marked increase was produced in the prothrombin time values of plasma of guinea pigs through the administration of dicumarol, without any accompanying change in the whole complement titer of the serum or in its relative concentration in the four major complement components.

Introduction

As outlined in our first paper (22), it is proposed in these parallel studies of complement and coagulative activity, to subject guinea pigs or other animals to different methods of treatment known to affect one or other of these properties, to determine the relative changes in each, and to ascertain whether these bear any apparent interrelationship. Dicumarol, because of its well recognized anticoagulative effect *in vivo*, was selected as one of the agents for study.

Literature Review

The fatal haemorrhagic disease produced in cattle through the ingestion of spoiled sweet clover was first described by Schofield (24). Physiological studies of Roderick (23) indicated a gradual and progressive decrease in prothrombin to be the cause of the delayed coagulability of the blood. Although focal necrosis was a common lesion in the livers examined there was little sign that other functions of the liver had been seriously impaired. Some years later, Quick (17) verified the fact that a decrease in prothrombin was involved. In 1941, Link and his associates undertook a chemical investigation of the toxic material, found it to be a dicoumarin compound, 3,3′-methylene*bis*(4-hydroxycoumarin), and succeeded in synthesizing it (4, 5, 26). Bingham *et al.* (3) observed that the prolongation in prothrombin time was not demonstrable until 24 hr. after the administration of dicumarol, and concluded that although the formation of prothrombin in the liver was inhibited, it was not until the amount already present in the blood had been used up that the effect of the drug could be detected. Lein and Lein (12) on the other hand, considered the action of dicumarol was not to inhibit the formation of prothrombin, but to lead to the production of an altered form of this element. This altered prothrombin was converted to thrombin by lipid- but not by protein-thromboplastic agents. Since the quantity of prothrombin in normal plasma is too

[1] *Manuscript received September 27, 1950.*

Contribution from the Division of Animal Pathology, Science Service, Dominion Department of Agriculture, Animal Diseases Research Institute, Hull, Que.

small to be differentiated by electrophoresis, it is not surprising that the changes in plasma after therapeutic dosage levels of this drug are not of sufficient magnitude to be detected by this technique (25).

The fact that complement midpiece and prothrombin were claimed by Fuchs (9) to be identical and that they both occur in Fraction III–2 of plasma separated by modern careful chemical methods (6, 7) suggested that such prothrombin-depleted bloods would be of particular interest in investigation of the relationship of complement activity and coagulation.

Methods

Treatment of Guinea Pigs

Forty guinea pigs, weighing approximately 500 to 700 gm., were divided into two equal groups and given the same diet which consisted of commercial rabbit pellets, grass, and roots. One group was fed 15 daily oral doses of 5 mgm. of dicumarol suspended in 2 ml. of a 5% glucose solution. The other group was not treated.

All of the guinea pigs were bled from the heart on the 3rd and 16th days of the experiment, that is, after the treated group had received 2 and 15 doses of dicumarol. Part of the blood was oxalated, centrifuged within 30 min., and the plasma removed carefully. The remainder was allowed to clot, the serum drawn off and centrifuged.

Coagulation Tests

The prothrombin time values of the plasmas were determined by the methods of Quick and of Howell (11) and by a modification (13) of the two-stage technique of Warner, Brinkhous, and Smith (28). For the last test, the plasma was diluted 1 : 25 and the fibrinogen prepared from sheep rather than from human blood.

Complement Titrations

The quantity of each serum required for 50% hemolysis of the standard quantity of maximally sensitized sheep red cells was ascertained as in the first series of studies (22, 27). Titrations of the four major complement components, C′1, C′2, C′3, and C′4, were made by the method of Bier *et al.* (2). In this case the point of complete hemolysis was used as the basis of comparison and the titers expressed accordingly.

Results

Prothrombin Time Values

All three methods of determining the prothrombin time of the plasmas showed that the dicumarol treatment had produced a marked change in the coagulative activity of the blood. Although this effect was discernible after

TABLE I

COAGULATION TIME VALUES OF PLASMA OF DICUMAROL-TREATED AND UNTREATED GUINEA PIGS AS DETERMINED BY THREE TECHNIQUES

Bleeding	Treatment	No. of doses	No. tested	Quick			Howell			2-Stage					
										Conversion time			Clotting time		
				Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.
1	None	0	18	23–33	28.0	2.9	44–70	56.7	7.6	130–185	147	16.4	32–94	56.7	17.2
2	None	0	15	22–30	26.0	2.5	41–67	54.4	8.3	113–163	142	20.5	18–80	48.4	16.6
1	Dicumarol	2	19	26–44	32.0	4.5	47–128	70.7	20.6	142–373	199	58.4	74–705	154	147
2	Dicumarol	15	15	25–70	40.2	11.8	44–144	86.2	29.0	131–568	297	152	82–735	336	225

two doses it was much more pronounced after 15. The two-stage method suggested that these changes were extensive. The differences in the mean values for the treated and untreated groups were statistically significant.

To facilitate comparison of the results obtained by the three methods, the plasmas were divided into groups according to their Quick coagulation time values (Table II). Although both the Quick and Howell values were increased

TABLE II

COMPARISON OF RESULTS OF QUICK, HOWELL, AND TWO-STAGE METHODS

Number of plasmas	Series	Quick		Howell			Two-stage clotting time		
		Range, sec.	Mean, sec.	Mean, sec.	Ratio to Quick		Mean, sec.	Ratio to Quick	
					Range	Mean		Range	Mean
6	Untreated	20–24	23	50	1.8–2.8	2.0	49	1.4–3.3	2.1
19		25–29	27	57	1.6–2.6	2.1	53	1.3–3.6	2.0
8		30–34	31	57	1.6–2.1	1.9	53	0.6–2.1	1.7
6	Treated	25–29	28	58	1.2–2.5	2.0	89	2.5–3.8	3.2
15		30–34	31	66	1.5–2.6	2.0	136	2.6–8.0	4.2
5		35–39	36	75	1.6–2.4	2.0	230	2.1–10.6	6.4
5		40–44	43	112	2.4–2.9	2.6	473	4.0–15.3	11.2
3		45–70	56	122	2.0–2.3	2.2	619	7.0–14.1	11.5

in the dicumarolized animals, their ratio for individual plasmas still approximated 2.0, a ratio similar to that of the normal plasmas. Whereas the two-stage clotting times of normal plasmas were also about twice the respective Quick values, this ratio increased to a maximum of 15.3 for plasmas of the dicumarol-treated animals. The two-stage method therefore appeared to be detecting changes in the coagulative properties of the blood of treated animals that were not being revealed by either the Quick or Howell procedures.

In order to examine further the relationship between the three sets of values, curves translating the coagulation time values into percentage prothrombin were prepared. Pooled normal plasmas were diluted with 0.85% salt solution and tested by each of the techniques. The results are illustrated graphically in Fig. 1. As is shown in Tables I and II, the Quick coagulation time values for normal guinea pig sera varied from 20 to 34 sec., which according to Fig. 1 could represent a range of prothrombin concentration of 100 to 20%. The Howell prothrombin time values of normal plasma varied from 41 to 70 sec., which could correspond to a range of 100 to 10% prothrombin. The two-stage clotting time values showed normal limits of 40 to 90 sec., which from the chart could indicate a variation of 100 to 65% prothrombin. The two-stage method has also been found to be more sensitive in detecting an increase of prothrombin time in human plasma. Some of the suggested explanations of these differences are discussed later.

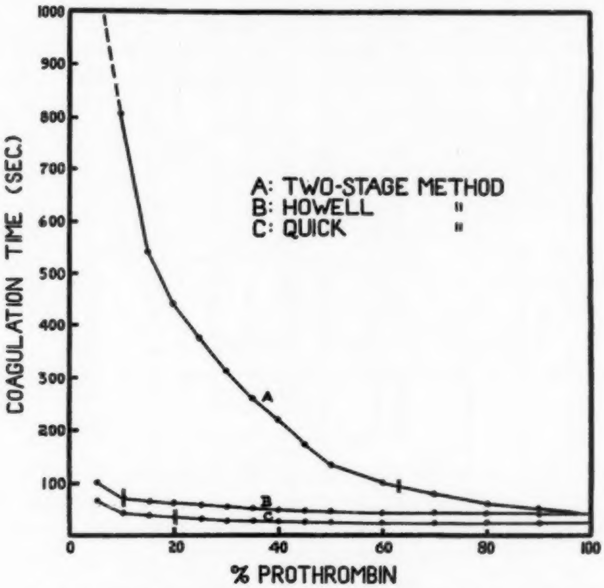

FIG. 1. Estimation of percentage prothrombin by three techniques.

Complement Titers

Unlike the prothrombin time values, the complement titers of dicumarol-treated guinea pigs did not differ significantly from those of normal guinea pigs. The mean complement titer after two doses of dicumarol was 1463 50% hemolytic units per ml. as compared with 1421 units per ml. for the controls. After 15 doses, the mean titer was 1566 units per ml. as compared with 1449 for the normal animals (Table III).

The C′1, C′2, C′3, and C′4 titers have also been included in this table. As usual C′4 was the component present in highest concentration with C′1 the next highest; C′2 was the lowest. The differences in the range and mean values for sera from treated and control animals were negligible.

Comparison of Coagulation Time Values and Complement Titers

Although it is evident from comparison of the data in Tables I and III that the marked changes in coagulative properties were not reflected in any corresponding change in complement titer, this is even more clearly indicated in Table IV in which all specimens have been arranged according to their two-stage clotting time values. Bleedings whose plasmas showed two-stage clotting times up to 735 sec. had serum C′, C′1, C′2, C′3, and C′4 titers comparable with those of bleedings whose plasmas showed two-stage clotting times of 18 to 49 sec.

TABLE III

TITER* OF SERA FROM TREATED AND UNTREATED GUINEA PIGS IN WHOLE COMPLEMENT (C′), AND EACH OF THE FOUR MAJOR COMPLEMENT COMPONENTS, C′1, C′2, C′3, AND C′4

Bleeding	Treatment	No. of doses	No. of guinea pigs	C′		C′1		C′2		C′3		C′4	
				Range, units	Mean, units	Range, units	Mean, units	Range, units	Mean, units	Range, units	Mean, units	Range, units	Mean, units
1st	None	0	18	1090–1670	1421	1000–5000	1688	450–1000	674	500–1000	794	1000–5000	3120
2nd	None	0	14	1390–1500	1449	1000–2500	1424	500–1000	746	500–2000	1043	2500–10,000	5000
1st	Dicumarol	2	19	1110–1700	1463	500–2500	1289	400–1250	700	400–1500	916	2000–5000	3740
2nd	Dicumarol	15	14	1610–1780	1566	1000–1670	1306	500–1050	729	1000–2000	1071	2500–10,000	5540

* *The titers of C′ are expressed in terms of the amount of complement required for 50% hemolysis; for C′1, C′2, C′3, and C′4 in terms of the amount required for 100% hemolysis.*

TABLE IV

COMPARISON OF MEAN COMPLEMENT TITERS AND TWO-STAGE CLOTTING TIMES

Two-stage clotting time, sec.	No. specimens	Mean titers (units/ml.)*				
		C'	C'1	C'2	C'3	C'4
18 to 49	13	620	1430	620	800	5000
50 to 99	30	620	1420	530	930	5500
100 to 199	11	590	1250	480	1110	6250
200 to 299	3	770	1000	460	670	7690
300 to 399	3	710	1430	440	900	6900
400 to 599	3	830	1110	620	830	6000
600 to 735	3	710	1250	619	1080	5000

* *For convenience in comparison, C' as well as C'1, C'2, C'3, and C'4 titers have been expressed in 100% hemolytic units.*

Discussion

That different methods of determining the coagulative activity of the blood may give different results is to be expected in view of the complexity of this phenomenon. In the Howell test, calcium is the only reagent supplied; in the Quick test, both calcium and prothrombin are added. The two-stage method, in which the plasma is diluted, partially avoids antithrombic and antithromboplastic effects (15, 28). Furthermore, a barbitone buffer is used to maintain a constant pH, and an excess of standardized fibrinogen and thromboplastin added to compensate for variation in these factors in individual samples. With so many variables controlled, the two-stage method might be expected to be a more sensitive indicator of fluctuations in prothrombin activity. Such indeed has been the experience of a number of investigators who have used these various tests in the control of dicumarol therapy (6, 10, 16). Olwin (15), however, has found one-stage methods of value in estimating the summative effect of the various clotting factors.

As has already been stated in the literature review, it was initially believed that dicumarol acted mainly on prothrombin (3, 5, 17, 23). More recently "accelerator-globulin", a factor in the globulin fraction of plasma and serum which accelerates the conversion of prothrombin to thrombin, has also been claimed to be affected (8, 14, 15). Owen and Bollman (16) found in dicumarol-treated dogs, that not only prothrombin but a factor important in its conversion to thrombin was decreased. Alexander *et al.* (1), likewise, found such blood to be low in prothrombin-conversion accelerator.

Quick and Stefanini (20, 21) have recently introduced another theory to account for the blood changes in dicumarol poisoning. This hypothesis is based on an earlier postulate of Quick (18) that prothrombin is a complex of three components, component A, component B, and a labile factor. Component A which corresponds to classical prothrombin, seems to exist in an active and in a precursor form; it does not diminish rapidly on storage but is affected by dicumarol. Component B may be concerned in the conversion

of prothrombin A precursor to the active state (19). The labile factor, which diminishes on storage, is not reduced by dicumarol treatment (20). In explanation of the differences in the prothrombin values obtained by the one-stage and two-stage methods, Quick and Stefanini suggest that in the two-stage method the conditions are such that all of component A is converted to the active form, whereas the one-stage method determines free or active component A only.

Irrespective, however, of the nature of the change in prothrombin and its associated factors, it is apparent from the results of the experiments reported above that marked changes can be produced in the activity of the complex without being reflected in any significant change in whole complement titer of the serum or its relative content in the four major complement components.

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PARALLEL STUDIES OF COMPLEMENT AND COAGULATION

III. THE EFFECT OF THE PROTEIN LEVEL OF THE DIET[1]

BY CHRISTINE E. RICE, PAUL BOULANGER, AND P. J. G. PLUMMER

Abstract

The complement and coagulative activities of the blood were studied in three groups of 20 guinea pigs each, receiving diets containing different amounts of protein, 2.3 to 26.7%. The mean complement titers were found to be lower and the prothrombin times longer in the group fed the diet containing the least protein, 2.3%. Of the four major complement components, the fourth, C′4, appeared to be most affected. Sera of the animals receiving less protein were lower in total protein content and had lower albumin – globulin ratios than sera from guinea pigs on the higher protein diets. No relationship was demonstrated between coagulation time and serum protein content.

Introduction

Since protein constituents of the blood are important in both coagulation and complement activity, diets low in protein might be expected to produce some disturbance in either or both of these activities. Reduced food intake, and more notably of protein, was indeed suggested as a possible cause of the lowered complement titers observed in a small proportion of the highly emaciated, scorbutic guinea pigs in our first series of experiments (15). The present paper reports the results of an investigation of this possibility. Series of guinea pigs of comparable weight were placed on diets adjusted to different protein levels and the complement titers of their sera and the coagulative properties of their plasma determined after a six-week interval. Protein analyses were made on representative numbers of individual sera from each dietary group.

The guinea pigs were not bled at the beginning of the experiment since it was felt that this would introduce another variable—the difference in the rate of restoration of blood proteins in individual animals. Instead, comparison of complement titers, prothrombin times and serum protein values for the animals on low-protein diets were made with the corresponding mean values for a group of animals of similar initial weight, maintained on a supposedly adequate diet during the same period.

Methods

Experiment I

On Nov. 28, 1949, 30 guinea pigs were weighed, distributed on the basis of weight into three groups of 10 each, and fed diets A, B (*a*), and C as described below. Five animals from each group were bled on Jan. 4 and five on Jan. 6.

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The weekly weight records showed that the majority of animals were losing weight during the period, especially those receiving Diet A; the mean weights of Groups A, B, and C animals were 762, 769, and 775 gm. respectively at the beginning of the experiment and 500, 575, and 689 gm. on Jan. 3.

Experiment II

The experiment was repeated on Jan. 9, 1950, with 30 more guinea pigs which were likewise divided into three groups of 10 each and fed diets A, B (*b*), and C. Three to five animals from each group were bled on Feb. 7 and 14. One animal in each group died during January. At the beginning of the experiment, the mean weights of the three groups were 720, 725, and 719 gm. respectively; on Feb. 13 their weights were 550, 706, and 761 gm., again indicating a considerable loss in weight in the Group A animals.

Diets

The basic diet for the guinea pigs in Group A was a mixture of three parts of oat hulls (containing 3.5% protein) and one part sugar, to which was added salt mixture, corn oil, and yeast extract to a concentration of 4% of each. For Groups B (*a*) and B (*b*) the same basic diet was used with the addition of 15 or 25% casein* respectively. Commercial rabbit pellets** formed the basic diet for Group C. All diets were supplemented by roots and the following vitamins: wheat germ oil,† 0.5 ml. per week; alphadol,†† 0.5 ml. per week; and ascorbic acid, 20 mgm. every two days.

Complement Titrations

The complement titers (17) of all sera were determined within two hours of collection and again 22 hr. later. They were estimated in terms of the minimum amount of complement required both for 50% and for complete hemolysis. Titrations of the concentration of C′1, C′2, C′3, and C′4 were made using test reagents prepared from guinea pig complement (1).

Coagulation Tests

The prothrombin time values were estimated for all plasmas by the methods of Quick and Howell (6, p. 101). A modified two-stage method (11, 18) was used in the determination of conversion and clotting time values.

* The "*Vitamin Free*" *casein*, salt mixture No. 2 U.S.P., and Brewers Yeast, U.S.P. XII, were received from Nutritional Biochemicals Corporation, Cleveland, Ohio. The Brewers Yeast was stated to contain in micrograms per gram: thiamine 150, riboflavin 65, niacin 475, pantothenic acid 125, pyridoxine 30, folic acid 22, biotin 2.2, choline 3600, and inositol 4500.

** "*Master Rabbit*" *Pellets*, from the Toronto Elevator Company Limited, Toronto, were guaranteed to contain a minimum of 15% crude protein, 4% crude fat, and a maximum of 11% crude fiber. The specific ingredients were stated to be as follows: dehydrated alfalfa meal, ground yellow corn, and/or hominy feed, alfalfa meal, soybean oil meal, linseed oilcake meal, feeding bone meal, ground wheat, wheat shorts, ground oats, wheat germ, wheat bran, ground limestone, 0.25% iodized salt, manganese sulphate, and fish oil fortified with vitamins A and D.

† *Wheat Germ Oil* (Vitamin "Rex") received from VioBin (Canada) Ltd., St. Thomas, Ont. was stated to contain 2.5 mgm. α-tocopherol per gm.

†† "*Alphadol*" from Ayerst, McKenna and Harrison Ltd., Montreal, Que., was stated to contain not less than 1500 International units of vitamin A and 400 International units of vitamin D per gm.

Serum Protein Tests

As a preliminary method of determining whether serum protein content was affected by the low protein diet, the well known cephalin–cholesterol flocculation test of Hanger (6, p. 236) was employed with one modification. Since in the dilution recommended, 1 : 20, normal guinea pig sera usually produced definite flocculation of the lipid suspension, the amount of serum was increased; with a 1 : 2 dilution of serum, stable suspensions were obtained.

The method described by Kibrick and Blonstein (5) for estimating the protein fractions in human serum was employed with minor modifications. Serum in 0.5 ml. amounts was added to 10 ml. of 15.75, 19.9, and 27.3% sodium sulphate solutions at 37° C. These correspond to final concentrations of approximately 15, 19, and 26% sodium sulphate. Infusorial earth was added and the mixtures let stand for one hour at 37° C. After passage through a No. 42 Whatman filter; the filtrates were diluted 1 : 63, 1 : 84, and 1 : 84 respectively, with 0.9% sodium chloride solution The nitrogen content of the diluted filtrates and of a 1 : 100 dilution of whole serum was determined by the turbidimetric method of Looney and Walsh (9). The amount of protein present was estimated by reference to a calibration chart. The calibration factor (turbidity reading in nitrogen equivalence) was determined by a micro-Kjeldahl method.

Results

It was initially proposed to place groups of guinea pigs on a basic diet composed of sucrose, salts, and corn oil supplemented by vitamins as recommended by Sober *et al.* (16). To this it was planned to add various proportions of purified or crude casein. Since our animals preferred to starve rather than eat these mixtures, various other possibilities were investigated before the basic ration of three parts of oat hulls and one part sugar was finally adopted. Although Diets B (*a*) and B (*b*) contained as much protein as, or more than Diet C, the general appearance of the animals and their slight loss in weight would indicate it to be less nutritious than the pellet diet given Group C.

The first section of the paper will discuss the results observed in complement titrations, coagulation tests, and protein assays; the second section will analyse the data obtained in these various tests in relation to each other.

I

Complement Titers

Table I summarizes the results of titrations of the four bleedings from the three dietary groups of animals. It will be noted that in both the first and second experiments, the mean amount of complement required for 100 and for 50% hemolysis, K'_{100} and K'_{50}, were closely comparable for Groups B (*a*), B (*b*), and C, and were within the range considered as satisfactory for routine complement fixation tests. The K'_{100} and K'_{50} values for the low-protein

TABLE I

COMPARISON OF THE K'_{100} AND K'_{50} VALUES FOR COMPLEMENTS OF GUINEA PIGS ON DIFFERENT DIETS

Date of bleeding, 1950	No. of sera	Dietary group	K'_{100}			K'_{50}		
			Range, ml.	Mean, ml.	σ, ml.	Range, ml.	Mean, ml.	σ, ml.
4/1 & 6/1	10	A	0.0016–0.0022	0.0020	0.00033	0.00090–0.00126	0.00112	0.00016
7/2 & 14/2	9	A	0.0016–0.0022	0.0019	0.00076	0.00067–0.00140	0.00106	0.00027
4/1 & 6/1	10	B(*a*)	0.0012–0.0022	0.0015	0.00033	0.00063–0.00110	0.00078	0.00017
7/2 & 14/2	9	B(*b*)	0.0012–0.0020	0.0015	0.00030	0.00065–0.00110	0.00079	0.00048
4/1 & 6/1	10	C	0.0012–0.0018	0.0014	0.00026	0.00065–0.00080	0.00073	0.00005
7/2 & 14/2	7	C	0.0012–0.0016	0.0014	0.00019	0.00065–0.00090	0.00071	0.00013

diet Group A, were significantly lower than those of the so-called "normal" Group C; in Experiment I the differences in their mean values were 5.6 and 6.8 times the standard error; in Experiment II, 4.5 and 2.5 times respectively. The course of the hemolytic reaction appeared similar for the low- and high-titer sera, as indicated by the fact that the ratio of the K'_{100} and K'_{50} values for individual sera roughly approximated 2.0 throughout.

As a general rule, the complement titers of fresh guinea pig sera compare closely with those of plasma from the same blood specimens. In the present studies, this appeared to be the case for blood specimens from the inadequately nourished animals as well as those from the control group. When plasma and serum samples from eight guinea pigs in each of the three dietary groups were tested in parallel, the titers obtained did not differ significantly. The ratio of the K'_{50} values for plasma and serum of Group A animals ranged from 1.08 to 1.37, mean 1.19; for Group B, 0.73 to 1.01, mean 0.88; and for Group C, 0.79 to 1.37, mean 0.98.

In addition to determining whole complement titers (C′), titrations were made of the content of the various guinea pig sera in components C′1, C′2, C′3, and C′4. The mean values for each as given in Table II show that throughout these were highest for Group C, and lowest for Group A. Of the individual components, C′4 seemed to be proportionately the most affected, C′3 proportionately the least. That C′4, the carbohydrate component, should be reduced to a relatively greater degree than either of the two protein components, C′1 and C′2, by the low protein diet, was somewhat surprising. It has also been shown to be the component most likely to be decreased in infectious diseases in man (3).

Coagulation Time

The methods of Quick and Howell, indicated the mean prothrombin times of the plasmas of guinea pigs on the basic oat-hull diet (2.3%) to be slightly longer than those of Group C. The more sensitive two-stage method detected

TABLE II

COMPARISON OF THE MEAN C′, C′1, C′2, C′3, AND C′4 TITERS* OF SERA OF GUINEA PIGS ON DIFFERENT DIETS

Date of bleeding, 1950	Dietary group	C′, units/ml.	C′1, units/ml.	C′2, units/ml.	C′3, units/ml.	C′4, units/ml.
4/1 & 6/1	A	526	1970	714	744	1820
7/2 & 14/2	A	560	1450	727	1040	2630
4/1 & 6/1	B(*a*)	625	2570	769	895	2920
7/2 & 14/2	B(*b*)	667	1490	767	1320	3330
4/1 & 6/1	C	714	2700	990	873	3840
7/2 & 14/2	C	709	1690	913	1330	5000

* *All titers are expressed in 100% hemolytic units per ml. of serum.*

even greater differences in the coagulative activity of the three dietary groups of animals; the differences between the mean clotting time values of Groups A and C in Experiments I and II, 27 and 16 sec., were 2.9 and 9.2 times the standard errors. In general, the animals of Groups B (*a*) and B (*b*) had prothrombin times longer than those of Group C, but shorter than those of Group A. There appeared therefore to have been no advantage from the standpoint of maintaining the normal coagulability of the blood, in increasing the amount of casein for the Group B animals from 15 to 25%.

Serum Proteins

The cephalin–cholesterol flocculation test, one of the commoner tests for disturbance in liver function, is designed to detect changes in the proportion of serum albumin and globulin and more particularly an increase in gamma-globulin. It was used therefore as a convenient preliminary test in determining whether the reduced protein content of the diet was being reflected in any change in the serum proteins. A pool of Group A sera gave definite flocculation, a Group B pool slight flocculation, whereas a Group C pool showed no tendency to flocculate the cephalin – cholesterol suspension. The van den Bergh test (6, p. 828) for bilirubin was negative on all three serum pools.

Protein values were then determined for individual sera from eight Group A guinea pigs, nine Group B (b), and four Group C. The results are summarized in Table IV.

Total Protein

The highest mean total protein values, 7.69 gm. %, were recorded for the four "normal" sera of Group C, the lowest for Group A, 6.53 gm. %. The values for Group B (*b*) sera varied from close to the minimum for Group A sera to the maximum for Group C; the mean value of 7.40 gm. % closely approximated the mean for Group C.

TABLE III

PROTHROMBIN TIME VALUES OF PLASMAS OF GUINEA PIGS ON DIFFERENT DIETS

Date of bleeding, 1950	No. of bleedings	Dietary group	Quick			Howell			Two-stage					
									Conversion			Clotting		
			Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.
4/1 & 6/1	10	A	24–39	30	5.7	43–109	77	23.7	155–192*	182	15.3	35–68	51	9.7
7/2 & 14/2	9	A	29–46	34	5.8	78–112	90	15.6	113–190	142	26.5	24–53	33	3.6
4/1 & 6/1	10	B(*a*)	26–36	30	3.9	42–81	64	12.3	113–153	136	16.3	20–49	39	9.7
7/2 & 14/2	9	B(*b*)	24–36	31	4.3	44–99	79	16.9	100–172	133	26.5	21–65	35	15.6
4/1 & 6/1	10	C	24–31	27	2.4	41–72	54	11.0	123–147	136	8.4	15–37	24	8.2
7/2 & 14/2	7	C	21–26	24	1.9	45–59	51	5.9	110–148	120	16.9	13–21	17	3.4

* *Eight of these 10 sera showed atypical coagulation, suggesting an insufficiency of fibrinogen.*

TABLE IV

PROTEIN VALUES OF SERA FROM GUINEA PIGS ON DIFFERENT DIETS

	Group A			Group B (*b*)			Group C		
	Range	Mean	σ	Range	Mean	σ	Range	Mean	σ
Total protein, gm.%	5.78–7.57	6.53	0.74	6.00–8.37	7.40	0.96	7.23–8.37	7.69	0.59
Total globulin, gm.%	2.54–3.27	2.92	0.29	2.42–3.73	3.13	0.48	3.13–3.62	3.33	0.27
Total albumin, gm.%	2.82–3.97	3.49	0.47	3.07–5.00	4.27	0.68	4.10–4.74	4.36	0.76
Albumin–globulin ratio	0.95–1.25	1.14	0.13	1.02–1.67	1.38	0.25	1.27–1.36	1.33	0.05
% of total globulin in									
15% Na_2SO_4 ppt.	29.3–52.1	41.1	10.2	30.2–48.6	39.4	6.3	35.4–48.9	42.7	6.5
19% Na_2SO_4 ppt.	34.8–29.8	16.7	7.0	5.3–24.7	11.5	6.5	14.5–20.0	17.0	2.7
26% Na_2SO_4 ppt.	0.8–57.7	42.2	10.6	40.6–62.0	49.1	6.5	26.6–41.0	40.3	6.0

Globulins

The total globulin values of Groups A and B (*b*) sera ranged somewhat lower than those of Group C, but the mean values for the three groups did not differ significantly from each other. Although the relative proportions of the globulins precipitated by 15, 19, and 26% saturation of sodium sulphate varied considerably for individual sera, the mean values, expressed in percentage of total globulin, were not significantly different for the three groups.

Albumin

The mean albumin values on the other hand were definitely lower for Group A sera, 3.49 gm. %, than for Group B sera, 4.27 gm. %, or for those of Group C, 4.36 gm. %. Again the Group B values ranged lower than those of Group C, although their mean values were relatively comparable.

As a result of this decrease in albumin, there was a significant depression below "normal" values in the albumin–globulin ratio of the Group A sera, mean ratio 1.14 as compared with 1.38 and 1.33 for Groups B and C. Some of the animals in Group B had had very low albumin–globulin ratios, minimum 1.02, some exceptionally high 1.67. Group C sera showed the least variation in this ratio ($\sigma = 0.05$).

Histopathology

At the completion of the experiment a number of animals in each group were sacrificed and a gross examination of their organs made: all appeared normal. Histopathological examinations were made of liver, kidney, and spleen from 23 guinea pigs. Kidneys of all animals and the spleen of all but one appeared normal. The livers of several animals showed some departure from the normal picture.

Group A

In three of the nine Group A livers examined the cytoplasm was rarefied, reticulated throughout except for a narrow band at the cell membrane. The liver of a fourth animal displayed similar but less extensive changes. There was no evidence of fatty degeneration and the nuclei were normal. Livers of the remaining five were histologically normal.

Group B

Four of the nine Group B livers examined showed some change, with the cytoplasm condensed about the membrane and reticular in the cell body. The degree of change varied considerably, being most severe in the fourth and least in the first. In no instance was nuclear damage apparent. The remaining five livers differed in no significant manner from the livers of the control group.

Group C

The livers from two of the Group C animals showed rarefied cytoplasm, one was slightly hypergranular, one was normal. The appearance of the first two livers was essentially similar to that of the fourth in Group A and the first in Group B. The changes were less extensive, however, than those observed in the other six atypical livers in Groups A and B.

One might conclude from these histological observations that even in the control group, the dietary or other conditions had not been quite optimal.

II

In this section a comparison is made, (*a*) of the complement titers with prothrombin times and with serum protein values and, (*b*) of the two-stage clotting times with complement titers and with serum protein data, in order to determine whether the three sets of values have any discernible relationship to each other.

Complement Titers and Coagulation Time

The blood specimens were divided into three categories on the basis of their serum complement titers and irrespective of diet. The coagulation time of the plasma from each specimen was noted, and the range and mean values for each of the three categories determined. In Table V it will be observed that

TABLE V

COMPARISON OF COMPLEMENT TITERS WITH COAGULATION TIME VALUES

Complement titer, units/ml.	No. of specimens	Quick			Howell			Two-stage clotting		
		Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ sec
1300–1760	27	21–39	29.1	4.6	41–99	64.2	14.3	13–65	28.0	13.0
1000–1250	13	24–46	30.0	7.0	41–109	70.8	25.7	16–55	35.6	13.1
700–999	13	26–39	30.4	4.1	42–112	78.5	22.5	23–68	43.6	12.3

the specimens in the highest-complement-titer category, 1300 to 1760 units per ml., had mean Quick prothrombin time values relatively similar to those of the lowest-complement-titer category, 700 to 999 units per ml. The difference between the mean Howell prothrombin times was somewhat greater.

The mean two-stage clotting time was however significantly longer for the group of specimens with the lowest complement titers than for the group with the highest complement titers.

Complement Titers and Serum Protein Values

Since the analysis of the serum proteins had indicated the only appreciable variation between dietary groups to be in total protein and albumin content, only these two sets of values were considered in relation to complement titers. Table VI shows that sera in the highest-complement-titer category also had

TABLE VI

COMPARISON OF COMPLEMENT TITERS, TOTAL SERUM PROTEIN, SERUM ALBUMIN, AND ALBUMIN–GLOBULIN RATIOS

Complement titer, units/ml.	No. specimens	Total protein			Albumin			Albumin–globulin ratio		
		Range, gm. %	Mean, gm. %	σ, gm. %	Range, gm. %	Mean, gm. %	σ, gm. %	Range,	Mean,	σ,
1300–1760	12	6.00–8.37	7.45	0.27	3.07–5.00	4.31	0.58	1.05–1.67	1.39	0.18
1000–1250	3	6.00–7.57	6.52	1.10	3.20–3.84	3.50	1.24	1.02–1.36	1.17	0.21
700–999	6	5.78–7.77	6.78	0.89	2.82–4.35	3.58	0.62	0.95–1.27	1.11	0.10

the highest mean total protein and albumin values, and correspondingly the highest albumin–globulin ratios. Conversely, sera in the lowest-complement-titer group had the lowest total protein, albumin, and albumin–globulin ratios. These differences were not statistically significant in the case of total protein but were so for albumin and for the albumin–globulin ratio (2.4 and 4.3 times the standard error).

Two-Stage Clotting Time and Complement Titer

The specimens were next divided into five groups on the basis of the two-stage clotting times of their respective plasmas. The group with the shortest clotting time, 13 to 19 sec., were found to have the highest mean complement titers, 1422 units per ml. Correspondingly, the group with the longest clotting times, 50 to 68 sec., had the lowest mean complement titers, 975 units per ml., Table VII. The mean complement titers of specimens in the first and second categories were not significantly different, and approximated "normal" values. This might be expected since a clotting time range of 13 to 29 sec., may also be considered as "normal". The differences between the mean complement titers of the third, fourth, and fifth categories and of the first were, however, statistically significant. Table VII, shows that, in general, an increase in Quick and Howell prothrombin times also accompanied the increase in two-stage clotting time and the decrease in complement titer.

TABLE VII

COMPARISON OF TWO-STAGE CLOTTING TIME VALUES AND COMPLEMENT TITERS

Two-stage clotting time, sec.	No. specimens	Prothrombin time						Complement titer		
		Quick			Howell					
		Range, sec.	Mean, sec.	σ, sec.	Range, sec.	Mean, sec.	σ, sec.	Range, units/ml.	Mean, units/ml.	σ, units/ml.
13 to 19	9	21–36	25.4	3.1	45–65	52.2	7.0	1110–1760	1422	191
20 to 29	13	24–39	28.2	4.7	41–108	66.4	23.2	710–1670	1331	243
31 to 37	11	24–36	29.9	4.1	41–97	70.8	16.8	770–1510	1175	296
40 to 49	12	26–36	31.5	3.1	42–112	74.4	20.9	910–1580	1190	308
50 to 68	8	26–46	33.4	7.9	57–109	83.4	18.4	790–1330	975	219

Two-Stage Clotting Time and Serum Protein Values

When the two-stage clotting time values were considered in relation to total protein, albumin, and albumin–globulin ratios, no correlation was demonstrated, Table VIII. The mean total protein value for the 13- to 19-sec. category was closely comparable with that of the 40- to 65-sec. category, as were their respective mean albumin–globulin ratios. Furthermore the range of values corresponded relatively closely in all four groups; all specimens with low protein values were in fact included in the two intermediate groups. Similarly, specimens with low albumin–globulin ratios occurred in all categories except the first.

TABLE VIII

COMPARISON OF TWO-STAGE CLOTTING TIME AND SERUM PROTEIN VALUES

Two-stage clotting time, sec.	No. specimens	Total protein			Albumin			Albumin–globulin ratio		
		Range, gm.%	Mean, gm.%	σ, gm.%	Range, gm.%	Mean, gm.%	σ, gm.%	Range	Mean	σ
13–19	4	7.23–8.37	7.69	0.59	4.10–5.00	4.42	0.45	1.27–1.48	1.36	0.09
20–29	7	6.00–8.37	7.18	1.09	3.46–4.74	3.93	0.68	1.02–1.36	1.22	0.15
31–37	6	5.78–6.97	6.28	0.59	3.07–4.35	3.45	0.59	0.95–1.48	1.23	0.32
40–65	3	7.57–7.77	7.64	0.14	3.97–4.74	4.35	0.46	1.09–1.67	1.34	0.42

Histopathology of Liver, Complement Titers, and Other Values

The mean complement titers of the four Group A guinea pigs that showed appreciable changes in liver cytoplasm were somewhat lower than those of the five with apparently normal livers, 830 units per ml. as compared with 1106 units per ml. The four animals in Group B in which the livers appeared somewhat abnormal, had mean complement titers of 1190 units per ml. in

comparison with a mean of 1471 units per ml. for the five without appreciable liver changes. The two animals of Group C that showed some deviation from normal in liver cytoplasm had complement titers essentially similar to those of the two animals with apparently "normal" livers.

No clear-cut relationships were demonstrable between these minor deviations from the "normal" in liver histology, and the variations in coagulative activity or in serum proteins, with the exception of a slightly higher proportion of globulin precipitated by 19% sodium sulphate (roughly corresponding to beta-globulin). In the eight animals in Groups A and B showing these liver abnormalities, the mean value for this globulin fraction was 19.3% of the total globulin as compared with 10.0% for the other nine animals in these groups.

Discussion

The foregoing studies with guinea pigs have demonstrated that when the protein content of their diet is reduced sufficiently to produce a considerable loss in weight, an appreciable decrease in the complement titer and coagulative activity of their blood may occur together with certain alterations in serum proteins, notably a decline in serum albumin. The fact that the specimens showing the most marked drop in complement activity were those displaying the greatest increase in coagulation time suggested that some common factor was involved. Although the fall in complement titer roughly paralleled the decrease in total serum protein and albumin, the prolongation in coagulation time bore no evident relationship to the changes detected in serum proteins. It would appear therefore that the common factor influencing complement and coagulative activities was not the same as that relating complement titers and serum protein values.

The relationship between complement titer and prothrombin time would seem most likely to be through C′1, in that this component is precipitated in the same fraction as prothrombin (2,4). However, since C′1 makes up only 0.6% of the total protein (14), even a 50% decline in this component could conceivably have occurred without its being detected by the relatively crude methods of fractionation employed in the present studies.

Although C′2, an alpha-globulin, and its accompanying carbohydrate, C′4, were the complement components showing the relatively greatest decline, it seems even more unlikely that such changes were detected by these methods since C′2 makes up an even smaller proportion of the serum proteins than does C′1, 0.18%. The apparent relationship between the decline in C′2 titer and serum albumin would seem to bear an analogy to the situation with certain enzymes, also alpha-globulins (2, 4), which likewise decline under conditions in which serum albumin is reduced (7, 8, 10, 12, 13), as if some common metabolic mechanism were affected.

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ON RAT SERUM LIPASE

II. THE EFFECT OF VARIOUS EXPERIMENTAL STATES[1]

By Jules Tuba and Robert Hoare

Abstract

Further studies are presented on the enzyme in rat serum which hydrolyzes tripropionin, tributyrin, and ethyl butyrate. There is an increase from low levels at birth to normal adult serum lipase activity by about the fourth week of life. The concentration of the enzyme remains quite constant from day to day providing the animals are not bled too frequently, i.e. not more often than once a day. Starvation for a week does not alter serum lipase activity. Additional studies confirm the earlier finding that the lipolytic activity of rat serum is elevated by alloxan diabetes, and it is shown that insulin, administered over a 24 hr. period, produces a partial return toward normal activity of the enzyme. Decreased serum lipase levels resulted from castration of male and female rats, injection of diethylstilboestrol into normal and gonadectomized animals of both sexes, and late pregnancy. These decreases were all significant, although some of them were transient. It appears that some profound hormonal disturbances may be responsible for these changes.

Introduction

The microtechnique described by Tuba and Hoare (6) for the estimation of rat serum lipase has obviated the need for sacrificing the experimental animals since sufficient blood may be obtained from their tails for determination of lipase activity. It is therefore possible to study at different time intervals the lipolytic activity of serum of animals subjected to a variety of experimental conditions. A preliminary report (5) has already been published on the increased activity of lipase in the serum of alloxan diabetic rats. This study will now be presented in a more complete form, together with the effect of other experimental factors on the activity of the enzyme.

Method

The simple titrimetric micromethod for the determination of serum lipase concentration described by Tuba and Hoare (6) was used. Enough blood was taken from the tails of the animals by "milking" to yield 0.2 ml. serum in each case, the amount required for a control and an experimental determination. Bleedings were always performed at 8 a.m. This regular time was chosen in case diurnal variations exist in serum lipase activity similar to those found by Tuba, Baker, and Cantor (4) for rat serum alkaline phosphatase. The existence of similar fluctuations in the lipolytic activity of rat serum could not be established because we found that frequent bleeding of the animals resulted in decreased levels of the enzyme. Consequently animals were never

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bled more often than once a day. Serum lipase activity was usually determined the day blood was taken, although the enzyme has been found to be stable for several days.

For most lipase determinations tributyrin was used as a substrate, but in some experiments tripropionin and ethyl butyrate were also used. The hydrolyses of these substrates, using veronal buffers, were carried out at the following pH values: 8.05 for tributyrin, 7.6 for tripropionin, and 7.2 for ethyl butyrate.

Enzyme activity was measured, at 37° C., by the difference between the titration values of the experimental and control tubes. The lipase activity of serum in units is equivalent to the number of ml. of 0.025 *N* sodium hydroxide required to neutralize the amount of fatty acid set free by the enzyme contained in 100 ml. serum in 30 min. from tributyrin or ethyl butyrate or in a 15 min. hydrolytic period from tripropionin.

The results were analyzed statistically, and for each treatment the standard error of the mean differences or differences of the means were estimated. The t values are given in each case. $P = 0.05$ is taken as the level of significance.

Serum Lipase Levels in Normal Rats

In a previous paper (6) we stated that for normal adult male rats, mean serum lipase activity in units per 100 ml. serum was 596 (S.D. = $\pm$101) for tributyrin, 858 (S.D. = $\pm$120) for tripropionin, and 734 (S.D. = $\pm$84) for ethyl butyrate. These levels remained essentially unchanged when additional animals were subsequently tested. It was also noted that no significant differences existed between male and female rat serum lipolytic activity.

The serum tributyrinase activity of a group of eight male rats was followed at intervals from two days to 35 days after birth. It was necessary for the first three bleedings to pool the blood obtained from the group in order to obtain sufficient quantities of serum. It is apparent from Table I that the

TABLE I

THE RELATIONSHIP OF AGE TO MEAN SERUM LIPASE (TRIBUTYRINASE) LEVELS IN EIGHT MALE ALBINO RATS

Age (days)	Lipase, units/100 ml. serum	Age (days)	Lipase, units/100 ml. serum
2	150	24	317
5	110	27	431
8	100	28	484
10	100	30	670
15	120	33	660
21	166		

concentration of serum lipase increases from low levels at birth to adult levels by about the fourth week. A similar relationship was found by Copenhaver, Stafford, and McShan (2) in their studies on liver esterase.

It was noted in the early stages of the investigation that several daily bleedings lowered the levels of serum lipase, but that serum samples could be obtained at intervals of one or two days without affecting the enzyme concentration. Consequently normal lipase activity was followed at two-day intervals to eliminate completely this factor. The results in Table II are averaged for groups of 10 rats and indicate that variations over a period of 11 days are within the normal ranges of the three substrates.

TABLE II

VARIATIONS IN LIPASE LEVELS OF ADULT MALE RATS AT TWO DAY INTERVALS

Lipase activities in units/100 ml. serum are the averages for 10 animals in each group

Substrate	Time in days					
	1	3	5	7	9	11
Tributyrin	695	612	590	630	620	632
Tripropionin	735	790	830	772	923	913
Ethyl butyrate	737	650	657	663	747	635

Serum Lipase Levels in Starvation

Six adult male rats were placed in the same cage. For one week they were supplied with tap water but deprived of food. Serum lipase levels were determined daily but because enzyme activities remained within the normal ranges of fluctuations given in Table II only the initial and final results are given below in Table III. It was surprising to find, in spite of the pronounced loss in weight which occurred during the week, that the enzyme values remained virtually constant. After the starvation period the animals were returned to the normal stock laboratory diet of Purina Fox Checkers, and as a result the weights were restored to their former levels, whereas lipolytic activities were slightly but not significantly increased above normal. The behavior of serum

TABLE III

SERUM LIPASE (TRIBUTYRINASE) LEVELS OF SIX ADULT MALE RATS STARVED FOR SEVEN DAYS AND THEN RETURNED TO STOCK DIET FOR SEVEN DAYS

Time	Lipase, units/100 ml.			Mean weight
	Range	Mean	Standard deviation	
0 days	470–620	554	±116	227
7 days' starvation	520–670	592	± 59	168
7 days after return to stock diet	540–750	667	± 69	232

lipase during the starvation period is in marked contrast to that of serum alkaline phosphatase, which has been extensively studied in this laboratory, and which may be profoundly and readily affected by a number of factors, particularly food intake (4).

Lipase Levels in Alloxan Diabetic Rats

The effect of alloxan diabetes on the hydrolysis of tributyrin by rat serum lipase has already been reported by us (5). Subsequently the action of the enzyme on tripropionin and on ethyl butyrate was followed during the development of the diabetic condition. The results of a typical experiment using a group of six male animals for each of the three substrates are presented in Table IV. By the 16th day the increases over normal enzyme activity are

TABLE IV

SERUM LIPASE OF ADULT MALE RATS FOLLOWING INDUCTION OF ALLOXAN DIABETES (SIX ANIMALS IN EACH GROUP)

Substrate	Days after alloxan injection	Lipase in units/100 ml.		
		Range	Mean	Standard deviation
Tributyrin	0	460–660	562	± 73
	4	480–860	622	±127
	8	790–1440	982	±236
	12	610–900	770	±114
	16	630–1010	840	±125
Tripropionin	0	660–900	760	± 81
	4	670–930	800	±100
	8	870–1130	1007	±110
	12	790–1070	933	±104
	16	850–1240	1003	±146
Ethyl butyrate	0	670–900	760	± 31
	4	870–1250	1088	±150
	8	1150–1390	1288	± 89
	12	910–1220	1045	±118
	16	900–1130	1053	±112

approximately 50% for tributyrin, 30% for tripropionin, and 40% for ethyl butyrate. These increases are highly significant with t values of 4.7, 3.5, and 6.1 respectively.

Altogether at various times lipase activity has been observed in over 50 alloxan diabetic rats and invariably an increase has been found in the rate of hydrolysis of all three substrates and this has been found to persist for several months.

The cause of the rise in serum lipase activity observed in alloxan diabetic rats has not yet been established. Since it has been established that food

intake does not normally affect this enzyme, it does not appear likely that the greatly increased food consumption of diabetic rats is concerned. However, experiments are in progress which may eventually clarify this matter.

Effect of Insulin on Lipase Levels of Alloxan Diabetic Rats

Six adult male rats which manifested the typical hyperglycemia and elevated serum lipase activity of alloxan diabetes were given food and water *ad lib.* Beginning at 8 a.m. each animal was given an injection of 0.4 units of crystalline zinc insulin subcutaneously every three hours for 24 hr. It was felt that this series of injections would be the minimum required to show an effect on the activity of the enzyme. It was known that rat serum lipase was slower to respond to alloxan injection than rat serum alkaline phosphatase which has been shown by Cantor, Tuba, and Capsey (1) to respond very quickly. In order to avoid any diminution of lipase concentration by overbleeding, serum was obtained only at the beginning of the experiment and one hour after the last of the series of nine injections, i.e., at 9 a.m. the day following the first injection. Table V indicates that the average blood sugar

TABLE V

THE EFFECT OF INSULIN ON BLOOD GLUCOSE AND SERUM LIPASE (TRIBUTYRINASE) LEVELS OF SIX ALLOXAN DIABETIC ADULT MALE RATS

Time in hours	Blood sugar, mgm./100 ml.		Serum lipase, units/100 ml.	
	Range	Mean	Range	Mean
0	372–500	457	680–1110	981
25	110–325	207	590–900	805

NOTE: *For Table V statistical analysis is based on mean differences and their standard error. The range of differences for the six animals lies between −70 and −210 units, the mean difference is −112 ± 21.4.*

value was lowered by about 50%, while the average lipase level was decreased by 12%. The decrease in the enzyme activity is highly significant, with a t value of 5.2. A duplicate experiment resulted in a 14% diminution of the enzyme concentration.

The Relationship of Serum Lipase to Sex Hormones

The lipase levels of a group of eight male rats were estimated immediately before castration and for 30 days postoperatively as indicated in Table VI. There was a decrease of about 30% in the mean lipase value on the fifth postoperative day and this was followed by a gradual return to normal lipolytic activity by about the 15th day. In comparing the means for the 5th and 10th days with that for zero time, the t values for the differences were found to be 6.0 and 4.4 respectively, hence the results are highly significant.

TABLE VI

THE EFFECT OF ORCHIDECTOMY ON SERUM LIPASE (TRIBUTYRINASE) LEVELS OF EIGHT RATS

Days after operation	Lipase, units/100 ml. serum		
	Range	Mean	Standard deviation
0	600–800	709	±70
5	420–650	499	±73
10	550–710	624	±52
15	670–800	729	±51
30	610–770	680	±52

NOTE: *Statistical analysis for Table VI is based on mean differences and their standard error.*

The above results were repeated with 10 rats and similar results were obtained. A depression of the mean lipase value by about 20% was observed on the 10th postoperative day ($t = 3.4$) and values returned to normal, but more gradually than with the first group.

During the course of these experiments with castrated rats, food consumption for the 24 hr. period before lipase determinations was always found to be normal, and the weights of the animals increased slightly. The altered lipase levels could not therefore be attributed to these factors, especially since the results in Table III indicate that they do not significantly affect the enzyme. Loss of blood during the operations was too little to lower the concentration of lipase in the serum of any of the animals.

There was a possibility that the use of sodium amytal as an anaesthetic could have induced a temporary lowering of lipase levels. However, injection of sodium amytal into normal rats in anaesthetic doses produced no diminution of serum lipase four hours afterward. Schütz (3) had noted that prolonged administration of barbiturates decreases the cholinesterase activity of various tissues, although single doses had no effect.

Ten normal adult female rats were ovariectomized and serum lipase levels were followed as with the castrate males. A significant diminution of the mean lipase value by about 20% ($t = 3.1$) occurred on the fifth postoperative day. Recovery of lipase activity was more rapid than for the males. Food consumption and weight changes during the experiment were normal and operative bleeding was slight in all cases, so again, as with the males, these factors could not account for the altered enzyme activity.

It seemed possible that operative shock, or some hormonal disturbance following gonadectomy could result in the transient depression of serum lipase activity observed with animals of both sexes. The effect of some hormones on castrated and normal animals of both sexes was investigated: some of the other factors are now under consideration.

Each of the hormones used with male rats was tested on five normal and five castrated animals. The hormones were injected subcutaneously daily

for at least 14 days, a period found by Tuba, Baker, and Cantor (4) to be sufficient to affect rat serum alkaline phosphatase. The daily doses of the hormones used were: 1.0 mgm. testosterone propionate, 0.1 mgm. oestradiol dipropionate, 0.1 mgm. diethylstilboestrol.

Neither testosterone propionate or oestradiol dipropionate produced any alteration from normal serum lipase concentrations in either normal or castrated rats and the results are not reported. Diethylstilboestrol, however, produced a significant fall in serum lipolytic action by the seventh day (*t* values were 5.4 and 6.0 for orchidectomized and normal animals respectively). The decrease of about 45% in the mean lipase activities of both normal and castrated animals persisted for the remainder of the injection period. It is seen in Table VII that recovery, after injections were terminated, was more rapid in the control group than in the orchidectomized animals.

TABLE VII

THE EFFECT ON SERUM LIPASE (TRIBUTYRINASE) OF DAILY INJECTIONS OF 0.1 MGM. DIETHYLSTILBOESTROL IN FIVE ORCHIDECTOMIZED AND FIVE CONTROL ADULT MALE RATS

Experimental state of animal	Days after first injection	Lipase, units/100 ml. serum		
		Range	Mean	Standard deviation
Orchidectomized	0	550–840	704	±120
	7	300–440	382	± 53
	14	260–560	410	±135
	21*	450–620	526	± 61
	32	410–590	488	± 69
Normal	0	530–800	708	±100
	7	360–480	414	± 44
	14	310–350	334	± 20
	21*	420–600	490	± 73
	32	520–670	590	± 60

* *Hormone injections discontinued after this day.*

The only hormone injected into female rats was diethylstilboestrol. Daily doses of 0.1 mgm. were injected subcutaneously into five control and five oophorectomized rats. Again, as with the males there was a significant fall in enzyme activity (Table VIII). The values of *t* on the 14th day of the experiment were 5.6 and 4.7 for the oophorectomized and normal female rats respectively. A gradual recovery of lipase activity followed the cessation of the injections.

Food consumption by all animals receiving diethylstilboestrol was lowered to somewhat less than half normal, as previously reported by Tuba *et al.* (4). The results given in Table III would, however, rule out this as a cause of diminished lipase activity. There does appear to be some profound metabolic disturbance reflected in altered lipase levels initiated by disturbances in the

TABLE VIII

THE EFFECT ON SERUM LIPASE (TRIBUTYRINASE) OF DAILY INJECTIONS OF 0.1 MGM. DIETHYLSTILBOESTROL IN FIVE OOPHORECTOMIZED AND FIVE CONTROL ADULT FEMALE RATS

Experimental state of animal	Day after first injection	Lipase, units/100 ml. serum		
		Range	Mean	Standard deviation
Oophorectomized	0	570–810	685	±85
	7	350–530	470	±77
	14	380–500	442	±48
	21*	380–550	485	±73
	28	480–690	584	±85
Normal	0	630–810	726	±78
	7	450–610	536	±66
	14	400–550	510	±68
	21*	510–620	560	±64
	28	580–710	630	±60

* *Hormone injections discontinued after this day.*

normal hormone balance of the animal. This is further borne out by our studies with pregnant rats.

Vaginal smears were made daily from 10 adult female rats, and during oestrus they were placed with males, after bleeding for lipase estimations. Serum lipase levels were measured every five days. Only four animals of the group became pregnant and results for these are given in Table IX.

TABLE IX

THE EFFECT OF PREGNANCY AND LACTATION ON SERUM LIPASE LEVELS OF FOUR RATS

Days after onset of pregnancy	Lipase, units/100 ml. serum		
	Range	Mean	Standard deviation
0	490–670	583	±64
15	550–700	598	±59
20*	380–480	443	±74
25**	420–550	483	±48
30	520–640	568	±46

* *One day before birth of young.*
** *Fourth day of lactation.*
NOTE: *Statistical analysis for Table IX is based on mean differences and their standard error.*

During the latter part of pregnancy significant decreases appeared in the enzyme concentration of the rats. The value of t for the 20th day of pregnancy was 4.0. Lipase activity gradually returned to normal during early lactation.

A challenging characteristic of serum lipase is its lack of response to certain experimental factors such as partial or even total deprivation of food over comparatively long periods of time. This is a startling contrast to the rapid fluctuations of serum alkaline phosphatase activity, which in this laboratory has been shown to be affected by a number of dietary factors. The influence of sex, castration, and sex hormones were directly correlated by Tuba *et al.* (4) with the daily food intake. It is quite clear from the work reported above that such a correlation does not exist for serum lipase. Some of the depressions in lipase activity are transient, but even these persist too long to be accounted for on the basis of blood loss. Until further experiments are completed, one must assume that the lowered serum lipase activity observed after gonadectomy, and following injections of diethylstilboestrol, as well as during late pregnancy, are associated with disturbances of the normal hormone balance of the animals.

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